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CHAPTER 4

CLINICAL APPLICATIONS

DNA markers for the diagnosis of Wilson disease

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Abstract. Wilson disease is an autosomal recessive disorder of copper transport. The diagnosis of Wilson disease is usually made by measuring urinary copper output, serum ceruloplasmin concentration and liver copper concentration. However discrimination between heterozygotes and patients by conventional methods can be difficult. Since the gene has been assigned to chromosome 13 at 13q14-q21, DNA markers from this region can be used as a diagnostic aid.

We have investigated ten markers from the 13q14-q21 region in twelve families for which the diagnosis was well established, to confirm reported linkage results. These markers were also tested in three additional families, in which a sib of the index case had ambiguous results with conventional biochemical assays.

Our linkage results are similar to those of Middle Eastern families, supporting the hypothesis of a single disease locus. In the three families studied to establish the diagnosis, we confirmed the affected status of one sib. In the second family, we demonstrated that a presymptomatic sib, initially thought to be affected, is actually a heterozygote. In the third family, we could not determine if the sib in question is a heterozygote or an affected homozygote, given the markers presently available.

DNA markers can now be used as an aid for discriminating between patients and heterozygotes within families, provided an index case with Wilson disease is available and markers are informative. In some families, a larger number of close markers is required for definitive diagnosis by DNA methods. DNA marker studies will be particularly important when biochemical results are ambiguous.

Introduction

Wilson's disease is an autosomal recessive disorder of copper transport, characterized by copper deposition in the liver, and secondarily in the brain, cornea and kidneys[1,2]. The copper accumulation is caused by a decreased biliary copper excretion, but the underlying biochemical defect is unknown. Patients can usually be diagnosed by an increased urinary copper excretion, an elevated liver copper concentration, a serum ceruloplasmin concentration of less than 200 mg/l and the presence of corneal copper deposits (Kayser-Fleischer rings)[1,2]. When diagnostic uncertainty still exists, the incorporation of copper radioisotope into serum ceruloplasmin is considered to be the definitive diagnostic test[2]. Nevertheless it is sometimes difficult to distinguish between patients and heterozygotes, especially when the diagnosis is considered in a young patient whose copper accumulation is not yet sufficient to cause markedly abnormal laboratory results. Early diagnosis is important so that treatment can be initiated before tissue damage occurs[3,4]. Treatment usually involves the removal of copper from tissues by the chelating agents penicillamine or triene, although the use of zinc has been recently advocated[5,6]. Erroneous diagnosis of a heterozygote as a patient results in a lifetime of unnecessary medication, with its possible side effects.

The gene for Wilson disease has been assigned to chromosome 13 through linkage with the erythrocyte enzyme esterase D^7 and subsequently with other markers in the 13q14-13q21 region[8,9]. These polymorphic DNA-markers (restriction fragment length polymorphisms, RFLPs) have been used for carrier detection[10,11], and could be useful for the diagnosis of presymptomatic patients[11,12].

We report the application of this approach to three individuals for which the diagnosis of Wilson disease was uncertain, and in one case have reversed the initial diagnosis. Our observations demonstrate the importance of this approach as an aid to diagnosis.

Methods

Laboratory Analysis

Venous blood samples were collected in tubes containing EDTA, and high molecular weight DNA was prepared using the technique of Miller[13]. Three micrograms of DNA were digested with the appropriate restriction enzyme, according to the manufacturer's recommendations. The resulting DNA fragments were separated by electrophoresis in 0.6-1.0 percent agarose gels and transferred to a nylon membrane (Hybond-N, Amersham)[14]. DNA probes were labelled with [α-^{32}P]dCTP (Amersham) using a random priming kit (Pharmacia). Hybridizations were carried out at 65°C in 7% sodium dodecyl sulphate/0.5 M sodium phosphate, pH7.2/10mM EDTA/1% bovine serum albumin[15]. Filters were washed under conditions of high stringency (45mM sodium chloride, 4.5 mM sodium citrate, and 0.1% sodium dodecyl sulphate at 60°C) and autoradiography with dual intensifying screens and Kodak X-ray films was performed at -70°C for one to seven days to reveal restriction fragment lenght polymorphisms.

Families used for Linkage Analysis

A total of 12 Canadian families of northern European ancestry (mainly British and French) were used to study the linkage between the Wilson disease locus and ten markers at nine loci at 13q14-q21 (Table 1). In these families, 18 individuals were affected and 33 sibs were normal. The diagnosis of Wilson disease in the patients was based on a serum ceruloplasmin below 180 mg/l, a urinary copper excretion above 0.6 μmol/24 hr, and a liver copper above 250 μg/g dry liver tissue. Genetic linkage analysis was evaluated by pairwise testing of marker and disease locus using the computer program LIPED[16].

Table 1. Description of the markers used in this study.

Locus	Probe	Map location	Enzyme	Alleles
D13S22	pG14E3.8	13q14	HindIII	2.3, 2.0
ESD	pEL-22(3')	13q14	ApaI	8.6, 7.0
RB1	p68RS2.0	13q14	RsaI	8 alleles(2.0-1.5)
RB1	p88R0.6	13q14	XbaI	7.0, 5.5
D13S31	pCR1324	13q14	1)TaqI	6.7, 4.6
			2)PvuII	7.3, 5.5+1.8
D13S55	CRI-R214	13q14-21	TaqI	8.9, 6.0
D13S26	pH2-10	13q21	1)HphI	2.8, 2.0
			2)BclI	6.3, 5.6
D13S39	WC25	13q21-22	MspI	4.2, 2.4, 2.2
D13S41	WC83	13q21-22	TaqI	8.5, 2.4
D13S12	pG18E2.1	13q21	MspI	8.8, 4.4
D13S2	p9D11	13q22	MspI	15.0,11.0, 10.5
D13S4	p1E8	13q22-31	MspI	10.1, 7.4

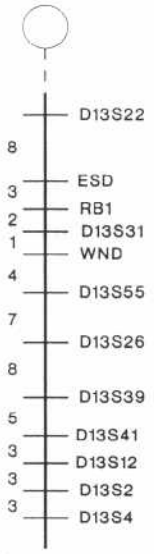

Figure 1. Genetic map of the long arm of chromosome 13. Numbers to the left represent distance between loci, expressed as centimorgans, and are an average of male and female distances (from refs. 17 and 18).

Families for Diagnostic investigation

Parents and children of three families were typed for twelve polymorphic DNA markers which have been mapped to chromosome 13 (table 1). These markers cover a total distance of approximately 50 centimorgans (cM), and flank the Wilson disease gene[17,18] (Fig. 1). Our results using rodent x human hybrid cell lines are compatible with this marker order[19].

Table 2. Results of laboratory investigations in three families.

Family	No	Age* (yrs)	Serum ceruloplasmin** (mg/l)	Serum copper** μmol/l	Urinary copper** μmol/24 hrs	Liver copper** μg/g dry
1(W46)	II-1	10	ND***	51.9	ND	ND
	II-2	9	110	5.4	12	ND
	II-3	7	181	10.0	0.7	ND
	II-4	3	281	ND	ND	ND
2(W6)	II-2	20	30	3.5	0.6	692
	II-3	9	ND	ND	ND	2190
	II-4	11	60	5.4	1.3	1137
3(W43)	II-1	19	70	4.0	2.0, 3.5, 1.6	ND
	II-2	17	30	4.4	4.8	ND
	II-3	9	250	ND	ND	ND

*Age at which stated results were obtained. **Normal values: serum ceruloplasmin :180-450 mg/l; serum copper 10.5-23.0 μmol/l; urinary copper: < 0.6 μmol/24 hrs; liver copper: 50 μg/g dry tissue. ***ND: not determined.

Family 1 (W46)

The proband (Fig. 2, II-1) in this family presented with fulminant hepatic failure at the age of 10 years and died within a few days. Because of the presence of Kayser-Fleischer rings Wilson disease was suspected clinically. This was proven at autopsy: the typical histopathological changes of Wilson disease were seen in the brain.

At that time there were two other children in this family: a brother aged nine years and a sister aged three years. The boy (II-2) had hepatosplenomegaly but no Kayser-Fleischer rings. Serum ceruloplasmin was low and urinary copper excretion was elevated (Table 2). Liver biopsy showed steatosis, chronic active hepatitis and early cirrhosis, with a positive stain for parenchymal copper. Wilson disease was diagnosed and treatment with penicillamine was started. In contrast, the sister (II-3) appeared well and had no hepatosplenomegaly. Her serum ceruloplasmin was low at 120 mg/l, but urinary copper excretion was normal. Thus it was unclear whether she was a heterozygote or a patient, and she was re-evaluated at yearly intervals with no change in findings for several

years. At the age of 7 years she presented with a slightly changed clinical picture. Although she had been well, on physical examination hepatomegaly was noted, as well as several spider angiomata. Serum aspartate aminotransferase (AST), previously normal, was 77 U/l (normal < 36 U/l). Serum ceruloplasmin was low. The urinary copper excretion was just above normal, but not in the range usually seen in Wilson disease (Table 2). Kayser-Fleischer rings were not detected by slit lamp examination. Liver biopsy showed mild steatosis on light microscopy. On electron microscopy, however, multiple features compatible with a diagnosis of Wilson disease were found. On the basis of these studies, Wilson disease was diagnosed. DNA marker studies were initiated to verify this diagnosis.

A fourth child, born after the proband died and presently aged 3 years, was also evaluated. Since serum ceruloplasmin was normal (Table 2), this child appeared to be unaffected. Marker studies were used to determine his genotype.

Family 2 (W6)

The proband (Fig 3., II-3) died at 9 years of age from acute hepatitis. An autopsy revealed a cirrhotic liver. Liver copper was elevated (Table 2). An older sister (II-1) had died six years previously of liver failure, diagnosed as acute hepatitis, at age 15 years. Based on family history, clinical symptoms and the marked elevation of liver copper, a diagnosis of Wilson disease was made for II-3, and was also assumed to be the cause of death in the older sister. The other two sibs (II-2 and II-4) were then investigated. Both had low serum ceruloplasmin concentrations (Table 2). Urinary copper excretion was normal in the sister, borderline in the younger brother. Liver copper was slightly elevated in both. Neither had Kayser-Fleischer rings. Tests of liver function were normal in II-2, but the brother (II-4) was thought to have a slightly enlarged liver (3 cm below the costal margin). AST was elevated at 50 U/l. As a final diagnostic test, incorporation of a tracer dose of intravenous radioisotopic copper (^{67}Cu) was studied as described[20]. No incorporation into ceruloplasmin was measured in either. The brother was diagnosed as affected, based on clinical signs and biochemical results. II-2 was also diagnosed as affected, but with less certainty because of the lack of clearly abnormal biochemical results and clinical signs,

particularly when older than her affected sibs. Conclusions from the ^{67}Cu studies also raised some doubt because a paternal aunt, who was in good health at 65 yrs of age, and who, with a ceruloplasmin concentration of 120-180 mg/l, was probably a heterozygote, also showed no detectable incorporation of radioisotopic copper into ceruloplasmin. Penicillamine was prescribed for both children. The sister remained on penicillamine until 30 yrs of age, then stopped during her second pregnancy. She was monitored regularly and since no clinical signs developed and urinary copper excretion and liver functions remained normal, did not resume penicillamine. The brother consistently refused medical follow-up and penicillamine therapy, except for sporadic medication from 15 to 17 years of age. Both sister and brother were recontacted at 47 and 37 yrs of age respectively, for the study of DNA-markers.

Family 3 (W43)

The proband in this family (Fig 4., II-2), a female, presented with a movement disorder at the age of 16 years. Kayser-Fleischer rings were noted on examination. A liver biopsy showed acute cellular degeneration and steatosis. Laboratory values, with a low ceruloplasmin and a high urinary copper excretion (Table 2), were indicative of Wilson disease and consequently penicillamine therapy was started.

The patients younger sister (II-3) had a normal serum ceruloplasmin concentration (Table 2). However the older brother (II-1) had a low ceruloplasmin and slightly abnormal liver function tests (AST was 81U/l). He was initially placed on penicillamine therapy because he was moving to an isolated region. In an attempt to clarify the diagnosis a liver biopsy was performed at age 19 years. Microscopically a mild fibrosis was seen, but no features specifically diagnostic of Wilson disease. Penicillamine was stopped after six months. Urinary copper excretion, determined from 4 weeks to 6 months later, was consistently just above normal (Table 2). Because the diagnosis was based only on a low serum ceruloplasmin, mildly abnormal liver function tests and an abnormal, but non-specific hepatic histology, some doubt existed regarding the diagnosis. DNA marker studies were therefore performed to aid in diagnosis.

Table 3. Two point linkage with the Wilson disease locus.

	Recombination (Theta)									
LOCUS	0.0	0.001	0.01	0.05	0.1	0.2	0.3	0.4	Z_{max}	$Theta_{max}$
D13S22	3.25	3.24	3.16	2.84	2.42	1.59	0.82	0.23	3.25	0.0
ESD	3.38	3.38	3.34	3.09	2.70	1.83	0.98	0.29	3.38	0.0
RB1	6.38	6.36	6.20	5.50	4.63	2.95	1.47	0.40	6.38	0.0
D13S31	2.76	2.75	2.67	2.29	1.82	0.98	0.40	0.09	2.76	0.0
D13S55	$-\infty$	1.14	2.04	2.29	2.04	1.39	0.62	0.16	2.31	0.04
D13S26	$-\infty$	0.69	1.64	2.09	1.98	1.35	0.70	0.20	2.09	0.06
D13S39	$-\infty$	-0.67	0.30	0.82	0.89	0.68	0.36	0.10	0.89	0.09
D13S41	$-\infty$	-1.60	-0.62	0.00	0.17	0.21	0.13	0.04	0.23	0.17
D13S12	$-\infty$	-2.19	-1.19	-0.47	-0.17	0.04	0.06	0.02	0.06	0.27

Results

Linkage studies

In our families of northern European ancestry, the tested markers in the 13q14 region (D13S22, ESD, RB, D13S31) showed no recombination with the Wilson disease locus (significant lod scores for all markers except D13S31, with a lod score greater than 3, or odds of greater than 1000:1 against observed results being due to chance)(Table 3). The markers localized in 13q21 (D13S55, D13S26, D13S39, D13S41, D13S12) yielded lower lod-scores, due to obligate cross-overs between these markers and the Wilson disease locus. These results are in agreement with those from larger series of families from the Middle-East and Europe[9,11,18,21]. Our selection of markers for the diagnostic studies is therefore appropriate.

Diagnostic Studies

In family 1 (W46), the diagnosis of Wilson disease in the brother (fig. 2, II-2) was made on the basis of a low serum ceruloplasmin and copper, an increased urinary copper excretion (Table 2), as well as clinical and pathological signs. In the sister (II-3), the diagnosis might be questioned

because clinical findings were subtle and the urinary copper excretion was only marginally increased, while the low ceruloplasmin could be due to the heterozygous state. However, her diagnosis of Wilson disease was confirmed using DNA markers, because she and the affected brother were genotypically identical for the total chromosomal region investigated (Fig. 2). Nine markers were heterozygous in one or both parents and therefore informative for recombination. The chance of having an undetected double recombination flanking the disease gene in II-3 is only 0.25% for the paternal chromosome, and 1.96% for the maternal chromosome. Thus the overall reliability of the diagnosis in this case is approximately 98%. Although there were no biochemical indications that the younger brother (II-4) is affected, it was not known whether he was heterozygote. With DNA-marker studies, he has now been diagnosed as homozygous normal. The summed chance of having an undetected double recombination including the Wilson disease gene on either paternal or maternal chromosome is approximately 2%. Therefore the overall reliability of excluding the heterozygous state is about 98%.

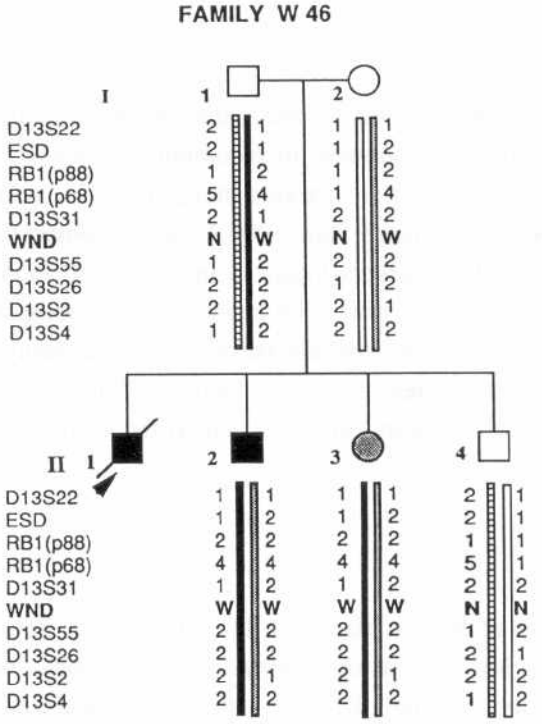

Figure 2.
DNA haplotypes in family 1 (W46). Only informative markers are shown. For each probe the longer fragment is designated 1, and the other 2. For the RB1 probe more alleles were recognized (see table 1). Inferred alleles for the Wilson disease locus (WND) are indicated by "W" for the mutant allele and "N" for the normal allele. The proband, II-1, indicated by an arrow, was deceased and no DNA was available.
Solid symbol: affected; clear: unaffected; stippled: status unknown prior to DNA study.
Chromosomes carrying normal alleles at the Wilson disease locus are cross-hatched (paternal) or open (maternal). Chromosomes carrying Wilson disease alleles are solid (paternal) and stipped (maternal).

In family W6, both living sibs have the same haplotype (Fig. 3). On reevaluation, both the sister is without clinical symptoms and has normal liver function tests, as well as the brother, who is now 37 years of age, and who has never had penicillamine therapy for a longer time. Based on this information, the early age at death of the two affected children, and the tendency for similar course of disease within families[20], we concluded that the brother, II-4, is not a patient, but is heterozygous. His sister, II-2, on penicillamine therapy for 10 years, is identical for all markers and therefore she is a heterozygote. So, finally, we could conclude that both sibs are not patients but heterozygotes and that the radiocopper studies had been misleading.

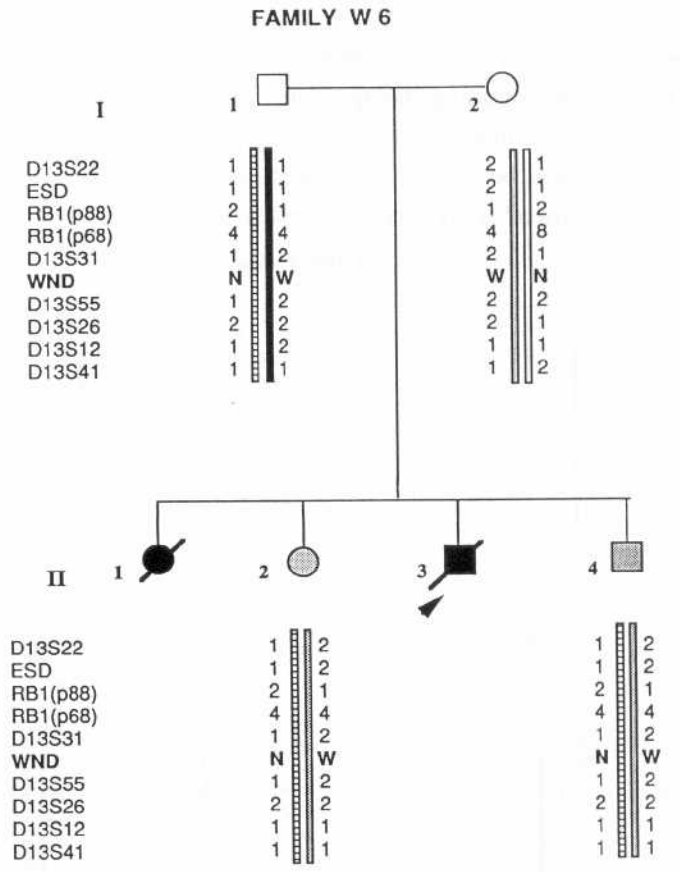

Figure 3. DNA haplotypes for family 2 (W6). Symbols are as for figure 2. An alternative for this haplotype is substituting the mutant allele and the normal allele on each chromosome pair.

In the oldest brother of the proband in family 3 (W43), a provisional diagnosis of Wilson disease was made, mainly because of a low cerulopasmin. Repeated assays of urinary copper excretion after he had ceased taking penicillamine for 4 weeks to six months were not extremely abnormal (Table 3). As is clear from Fig. 4, the subject (II-1) and his affected sister (II-2) are genotypically non-identical. However a recombination event has occurred in the proband near the Wilson disease locus. The two possible interpretations of the haplotype are shown in Fig.4. The chromosome on which the normal or Wilson disease allele occurs is exchanged in the mother to provide the alternate interpretation. Since we have no marker closer to the Wilson disease gene at present, we cannot determine whether the brother is a patient or a heterozygote. At present he is free of symptoms of the disease. Because of the uncertainty, both by biochemical assays and by DNA markers, the final diagnosis has been postponed. Rather than stopping therapy completely, he has been placed on zinc therapy, which is considered less likely to have side effects than penicillamine[5,6]. He will be closely monitored and his liver function and urinary copper excretion followed at six month intervals.

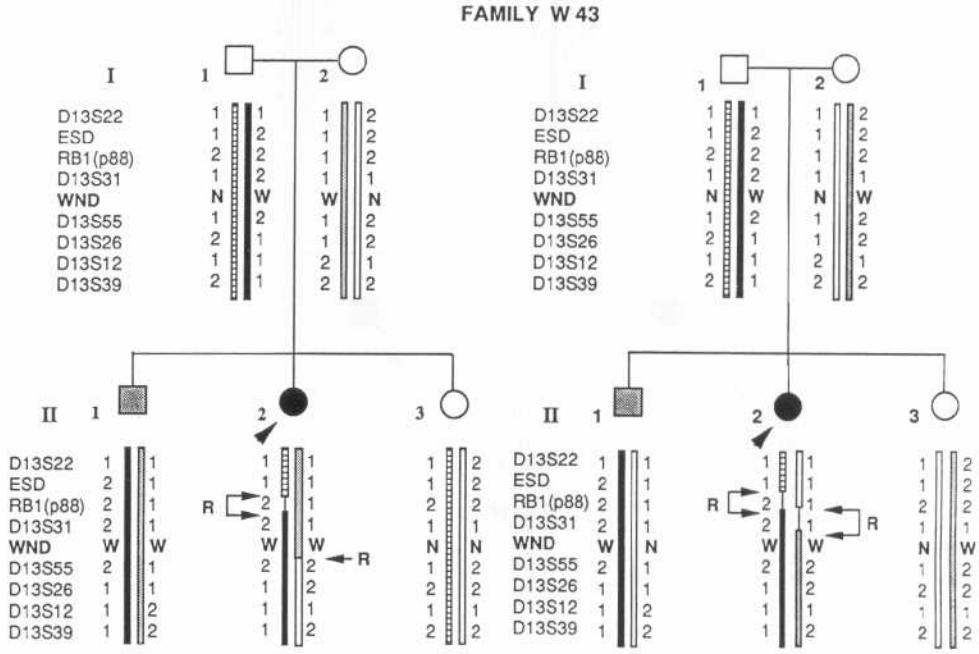

Figure 4. DNA haplotypes for family 3 (W43), showing alternative interpretations. R beside arrow(s) indicates location of recombination events. Other symbols are as for figure 2.

Discussion

The devastating hepatic and neurological symptoms of Wilson disease are caused by copper deposition in brain and liver, due to an unidentified defect in biliary copper excretion. Life-long treatment with copper-chelating agents, particularly penicillamine, has proven to be effective in achieving a negative total body copper balance. On such treatment, most patients become asymptomatic and it has therefore become the standard treatment for this condition[1,2]. When treatment is given to presymptomatic patients, tissue damage is prevented and the overall course is favourable[3,4]. An accurate diagnosis for presymptomatic sibs of an index-case is thus essential. If a heterozygote or normal unaffected individual is treated with penicillamine, serious side-effects may result. In patients who do not have Wilson disease, for example rheumatoid arthritis or biliary cirrhosis, side effects occur in about 33%[22]. Even for patients with Wilson disease, up to 20% experience side effects. Half of these are severe systemic lupus erythematosus or immune-complex-nephritis[23]. Therefore penicillamine should not be given to unaffected individuals.

Distinguishing patients from heterozygotes is sometimes extremely difficult. Heterozygotes may show some of the biochemical characteristics of patients, particularly a low serum ceruloplasmin concentration, which is characteristic of heterozygotes in some families previously designated as "atypical"[20]. An isolated low serum ceruloplasmin therefore cannot be interpreted as diagnostic for Wilson disease[1,2,20]. Instead it is recommended that the diagnosis of asymptomatic patients be based on a combination of a low serum ceruloplasmin, an increased urinary copper excretion and an increased hepatic copper concentration on liver biopsy[1-3]. This latter procedure is invasive, carries a small risk and can give unreliable results if there is any sample contamination. Likewise the administration of copper radioisotopes to study copper incorporation into ceruloplasmin (absent in Wilson disease) also poses potential risks and may be unreliable in individuals with a low serum ceruloplasmin concentration[12,24]. This is also demonstrated in Family W6, which shows that incorporation of copper into ceruloplasmin may not be detectable in heterozygotes with a low serum ceruloplasmin concentration. This family is similar to another family we have described, in which very low ceruloplasmin concentrations, lack of detectable incorporation of copper, and a modest

elevation of liver copper were characteristic of some of the heterozygotes and lead to erroneous diagnosis[24]. This method should therefore be used with caution in making a diagnosis.

The development of DNA diagnosis of Wilson disease, therefore, is of considerable importance[18]. Diagnosis using DNA markers potentially allows a clear distinction between patients and heterozygotes in families with Wilson disease. These advantages are illustrated by family 1 (W46), in which a diagnosis of affected was confirmed, and by family 2 (W6), in which the initial diagnosis of affected was reversed in one sib by DNA markers studies. However, family 3 (W43) illustrates the limitations of this approach. Because of a recombinant event in the proband, either of the two interpretations given in Fig. 4 will fit the marker results equally well and a final diagnosis for the brother cannot be made at present. When more very close markers are obtained it may be possible to do so.

Until the gene and its mutations are identified, there must be an index case in whom an unequivocal diagnosis of Wilson disease has been made. This patient's chromosome markers serve as a reference for identification of the disease-carrying chromosomes. When the chromosomes of the sibs are then studied, the chromosomes inherited from each parent can be identified, indicating whether or not the disease gene has been inherited. The sibs can be classified as patients, heterozygotes or homozygous normals. Furthermore, our results also show that with this approach 100% reliability cannot be obtained, because the diagnostic method is based on the use of flanking markers and there is always a chance of an undetected double recombinant, especially when only a few loci in the region of interest are informative. However the chance of having a double recombinant can be calculated and reliability estimated as we have done. Reliability will exceed 95% in most cases, because a large and growing number of markers on both sides of the Wilson disease gene is available, with the highly informative retinoblastoma marker close to the gene on the centromeric side.

Another potential source of error in the diagnosis of Wilson disease by linked markers would be the existence of more than one gene causing Wilson disease. There is some clinical evidence pointing to genetic heterogeneity, especially the observation that the affected members of a given family display similar clinical and biochemical phenotypes, while there is considerable

phenotypic heterogenity between pedigrees[20]. This variation could result from different mutations in the same gene, or from the action of environmental factors such as copper intake. If there were more than one gene causing Wilson disease, DNA diagnosis would be impossible at present. However current data argue against the existence of more than one gene. Wilson disease is localized on the same part of chromosome 13 in all populations studied to date: Middle Eastern[7-9,18], Northwest European[21], Italian[11], white American[9,18,25] and the Canadian (northern European ancestry) families studied in this report. Therefore the chance of having more than one gene causing Wilson disease in these diverse populations is remote. Nevertheless before this method is applied to individuals with a different genetic background, the localization of the Wilson disease gene in that particular population must be confirmed.

Ultimately the study of markers in the vicinity of the Wilson disease gene will lead to the isolation of the gene itself. This should lead to a real understanding of the pathogenesis of Wilson disease based on the gene function. It should also define the basis for the heterogeneity in clinical presentation and course of the disease. When the gene is isolated the diagnosis of Wilson disease will also be possible by DNA methods in patients with liver or neurological disease without a family history of Wilson disease.

In summary, we have reported here the development of a molecular genetic approach to the diagnosis of Wilson disease. This has important implications for identifying presymptomatic patients and for distinguishing between patients and heterozygotes in families with an index case of Wilson disease. This method can simplify the diagnostic evaluation of sibs of patients with Wilson disease in those families in which markers are sufficiently informative.

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